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Effect of silymarin alcoholic extract on surgery-induced intraperitoneal adhesion in rats

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Aim: Adhesion formation is the greatest complication in abdominal surgery. *Silybum marianum* (*S. marianum*) extract contains phenolic compounds whit antioxidant activities, thus could reduce adhesion formation. The aim of the present study was to examine its effect on intraabdominal adhesion.

Patients and Methods: Thirty male Wistar rats were randomly divided into three groups. Groups A and B received 1 per cent and 5 per cent concentrations of the *S. marianum* extract, and group C (control group) received distilled water. After anaesthesia was administered, the abdominal wall was opened and three shallow, longitudinal and transverse incisions (2 cm in length) were made on the right wall of the abdomen. A 2 × 2 cm piece was removed from the peritoneal surface on the left side of the abdominal wall; 3 mL of *S. marianum* extract or distilled water was then administered into the abdominal cavity. Adhesion degrees were determined according to the Canbaz scale. Histopathological examination was also determined according to the severity of fibrosis and inflammation. Data analysis was performed using SPSS version16.

Results: There was significant difference in the adhesion formation between the groups ($P = 0.023$). The adhesion degree in groups A and B were significantly lower than that of the control ($P < 0.05$). In the histopathological examination, significant differences were observed between the control and extract-treated groups in term of fibrosis and inflammation ($P < 0.05$).

Conclusion: *S. marianum* extract has preventive effects on post-laparotomy intraabdominal adhesion. Therefore, through further clinical studies, *S. marianum* extract and its derived compounds might be used in humans for the treatment of these complications.

Key words: abdominal adhesion, laparotomy, rats, silymarin.

Introduction

Adhesions develop due to incomplete lysis of fibrin and cellular exudates resulting from peritoneal injury.[1] Abdominal adhesions are the most unpleasant complications of abdominal surgery.[2] Adhesions are a common cause of infertility that occurs as a result of surgeries, such as tubal surgeries and surgeries to remove fibroids.[3] Pelvic adhesions are responsible for 25 per cent of all cases of infertility.[2] Adhesions occur following 50–97 per cent of abdominal surgeries and 60–90 per cent of gynaecological surgeries.[1,2] It is also known as the most common cause of small bowel obstruction (65–80 per cent of cases), which can lead to life-threatening ileus.[2] Adhesion bands are the leading cause of pelvic pain.[4] Other adhesion-related complications include chronic pelvic pain, urethral obstruction, voiding dysfunction and difficulties in reoperation.[1]

Many potential preventive agents have been investigated, including heparin, dalteparin, fibrinolytic materials, crystalloid solutions, corticosteroids, hyaluronic acid, hydrogel, nonsteroidal anti-inflammatory drugs, progesterone, melatonin, methylene blue and calcium channel inhibitors.[5–9] Antioxidant compounds, such as vitamin E, have been shown to possess inhibitory effects.[10,11] Statins can also prevent abdominal adhesions because of antioxidant and fibrinolytic activity.[12] Erythropoietin and melatonin have antioxidant properties and reduce oxidative stress and abdominal adhesions.[13] In their study, Scott-Coombes *et al.* showed that a peritoneal injection of superoxide dismutase and catalase was effective against oxygen-free radicals and reduced inflammation and subsequent adhesions.[14] In

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several cases, plants have been reported to have antioxidant activity and be effective in intraabdominal adhesions.[15,16]

Milk thistle [*Silybum marianum* (*S. marianum*)] is a member of the Asteraceae family. Milk thistle is also called holy thistle, Mary thistle and Marian thistle. Milk thistle is a medicinal plant with spiny leaves and sticky white sap that grows 30 to 90 cm tall and reproduces by seed. This plant grows all over the world and is indigenous to Iran. Fruits or seeds of milk thistle contain flavonolignan, a polyphenolic compound that is used as a liver-protective agent.[17] These compounds include silybin, isosilybin, silychristin, silydianin and taxifolin, which are collectively called silymarin. Silymarin, which is the most active component, is largely responsible for the pharmacological effects of this plant, including antiinflammatory, antioxidant, anticancer and hepatoprotective effects. Silymarin is found in various parts of plant, but the maximum amount is present in the seed.[18,19] Flavonoids, such as silymarin (and particularly silybin), are known to possess potent antioxidant and free-radical scavenging activities.[20] Studies on patients with alcoholic cirrhosis have shown that silymarin increases superoxide dismutase activity in lymphocytes and red blood cells, thereby enhancing antioxidant effects.[21]

Given its antioxidant and antiinflammatory activities, the *S. marianum* extract might be useful as an intraabdominal adhesion inhibitor. The *S. marianum* extract could inhibit intraabdominal adhesion through the inhibition of collagen production and accumulation, as well as complete lysis of fibrin caused by free-radical and proinflammatory factors. The aim of the present study was to evaluate the phenolic content and antioxidant capacity of *S. marianum*, and to determine its effect on intraabdominal adhesions.

Methods

This study was approved by the Shahrekord University of Medical Sciences (SKUMS) Ethics Committee (ethical code: 6-7-92) and was conducted at the Medical Plants Research Center of SKUMS. We attempted to respect all ethical principles of working on laboratory animals to impose the lowest stress on them.

Extract preparation

The maceration process was used for extraction. For this purpose, 2 L ethanol (70 per cent) was added to a flask containing 100 g of dried powder of *S. marianum* seeds. After 48 h, the extract was filtered through filter paper and the pulp was squeezed to discharge. The extract was concentrated using a vacuum distillation until the volume was reduced to 20 mL. The concentrated extract was dried.[16] One per cent and 5 per cent concentrations of the extract were prepared using distilled water. The solution was sterilized by passing through a 0.2-μm filter.

Measurement of phenolic compounds

The phenolic compounds were evaluated equivalent to gallic acid using Folin–Ciocalteu colorimetry, with some modifications.[22] Different concentrations of standard gallic acid (12.5, 25, 50, 62.5, 100 and 125 ppm in 60 per cent methanol) were prepared; 0.1 mL from each sample was then transferred into a test tube, and 0.5 mL 10 per cent Folin–Ciocalteu was added as a reactive agent. The solutions were left for 8 min at room temperature, and then 0.4 mL 7.5 per cent sodium carbonate was added. The tubes were maintained for 30 min at the laboratory temperature and then assayed at three intervals by a UV spectrophotometer (UNICO, Dayton, NJ, USA) at a wavelength of 765 nm. To measure the overall phenol in the extracts, 0.01–0.02 g of the extracts was solved in 60 per cent methanol, reaching 10 mL; using the Folin–Ciocalteu method, the overall level of phenol was then measured. However, instead of using the standard solution, 0.1 mL extract solution was added. Finally, the overall phenol level was obtained from the read optical density in mg/g extract in gallic acid equivalent.

Measurement of flavonoid compounds

Total flavonoids were evaluated equivalent to rutin, using chloride aluminum colorimetry and futin methods with a slight modification.[23] First, different concentrations of standard rutin (25, 50, 100, 250 and 500 ppm) were prepared in 60 per cent methanol. Then, from each solution, 1 mL was transferred into test tubes and mixed with 1 mL 2 per cent chloride aluminum. Afterwards, 6 mL 5 per cent potassium acetate was added, and the optical density level was read after 40 min at a wavelength of 415 nm. The concentration levels of the standard solutions were assayed at three intervals. In order to measure the overall level of flavonoids in the extracts, 0.01–0.02 g of the extracts was dissolved in 60 per cent methanol, reaching 10 mL. Then, using chloride aluminum colorimetry, the total level of flavonoids was measured. However, instead of using the standard solution, 1 mL of the extract was added. The total flavonoid level was calculated in mg/1 g extract, equivalent to rutin.

Measurement of flavonol compounds

The total flavonol was also measured using chloride aluminum colorimetry and the rutin procedure; however, the optical density level reading was obtained after 2.5 h at a wavelength of 440 nm.[24]

Measurement of antioxidant activity

The β-carotene model was employed to measure the antioxidant activity of the extract.[25] A total of 0.5 mL chloroform, 5 mL β-carotene (0.2 mg), 20 mL linoleic acid (20 mg) and 0.2 mL Tween 40 were mixed in a suitable container and incubated at 50°C for 10 min in order to remove the solvent. The solution was diluted with distilled water and mixed with 4 mL of aliquots in the following manner. The control solution was prepared with 0.2 mL ethanol and 0.2 mL of the extract sample with 0.15 mL ethanol. The optical density of the control group was recorded at t_0 and t_{90} at 470 nm, and similar to the standard group. The samples were incubated in a bain-marie at 50°C. The antioxidant activity was measured on the basis of the ability of the samples to prevent the washing of β-carotene. The antioxidant activity was calculated as follows: $AA = 100[1–(A_o–A_t)/(A°_o–A°_t)]$, where, A_o is the optical density at t_0, A_t is optical density of the sample at t_{90} and $A°_o$ and $A°_t$ are optical density values in the control samples at t_0 and t_{90}, respectively.

Selection and maintenance of animals

Thirty healthy, male albino Wistar rats, aged 3 months (200 ± 250 g), were used for study. The rats were randomly assigned into three equal groups, with each group consisting of 10 rats. The rats in intervention groups A and B were treated with 1 per cent and 5 per cent *S. marianum* extract, respectively. The rats in group C (control group) received only distilled water. The rats had no history of surgery or other medical interventions. The rats were kept on standard conditions at 23–25°C under a 12:12-h light/dark photoperiod, and were fed by standard pellet (Razi, Karaj, Iran).

Induction of adhesion lesions

All surgeries were done by one person. Both groups of rats were anaesthetized using a mixture of 20 mg/kg 10 per cent ketamine (Alfasan, Woerden, the Netherlands) and 2 mg/kg 2 per cent xylazine (Alfasan, Woerden, the Netherlands), administered intramuscularly. While anaesthetized, each rat was laid supine on a surgical table and the abdominal skin was shaved and disinfected with 10 per cent betadine in preparation for surgery. Under sterile conditions, a 2-cm incision was made on the midline of the abdomen, then then abdominal wall was opened and three shallow, longitudinal and transverse incisions (2 cm in length) were made with a scalpel no. 24 on the right wall of the abdomen. A 2 × 2 cm piece was removed from the peritoneal surface on the left side of the abdominal wall with surgical scissors. Then, to prevent the formation of peritoneal adhesions due to the presence of surgical suture material, four sutures at 1-cm intervals were placed using absorbable catgut. Fascia and skin were closed with four sutures at 1-cm intervals using a nonabsorbable silk. Finally, the given area of the skin was again disinfected, and the rats were left at a suitable temperature to regain consciousness. External sutures were removed on the 7th day of treatment under general anaesthesia.[16]

Treatment

The treatment period was 14 days, and the day of surgery was considered as the first day. Three millilitres of *S. marianum* extract at concentrations of 1 per cent and 5 percent was administered into the abdominal cavity of rats immediately after making lesions. The abdominal cavity was then sutured. The control group received only 3 mL of distilled water.

Macroscopic examinations

A second laparotomy was performed 14 days after making the lesions. For this, the abdomen of each rat was opened and adhesion scoring was performed by a blind observer. Total adhesion scores were calculated for each rat separately, according to the scoring method developed by Ahmet Canbaz (Table 1).[26]

Histopathological evaluation

Fourteen days after surgery, a sample of adhesion tissue from each animal was harvested, fixed in 10 per cent neutral buffered formalin and processed into paraffin and wax. The transverse incisions (5 μm thick)

Table 1. Macroscopic criteria for adhesion scaling

Score	No. adhesions
0	Without adhesion
1	1 thin adhesive band without vessels, and easily removable
2	2 thin adhesive bands without vessels, and easily removable
3	3 thin adhesive bands without vessels, easily removable
4	More than 3 thin adhesive bands without vessels, easily removable or scattered adhesive band

Table 2. Histopathological criteria for adhesion scaling

Score	Degree of inflammation	Degree of fibrosis
0	No inflammation	No Fibrosis
1	Giant cells, lymphocytes and plasma cells	Mild
2	Giant cells, plasma cells, eosinophils and neutrophils	Moderate
3	Inflammatory cell infiltration and micro abscess formation	Severe

were then made using a microtome fixed blade. All incisions of samples were stained by haematoxylin–eosin. Histopathological evaluations were performed by a blinded pathologist using a microscope (LABOMED, Los Angeles, CA, USA). Scoring of adhesion was performed separately based on the severity of fibrosis and inflammation (Table 2).[27]

Data analysis

Samples were numbered and data were recorded separately. Comparisons of data photographs were taken from abdominal adhesions. Data analysis was performed with SPSS version 16 (SPSS, Chicago, IL, USA) using Kruskal–Wallis and Mann–Whitney *U*-tests. $P < 0.05$ was considered statistically significant.

Results

The total phenolic content of *S. marianum* was 109.7 mg/g gallic acid equivalent; its total flavonoid content was 9 mg/g rutin equivalent/g and the total flavonol content was 5 mg/g rutin equivalent (based on dry extract). The antioxidant activity of the *S. marianum* extract was 35 per cent of β-carotene.

In the present study, all rats had normal preoperative activity and nutrition, and completely healed after operation. There were no signs of ascites, intraabdominal viscous fluid or mortality. After the laparotomy, intraperitoneal adhesions were formed in both groups because of cutting and killing of the rats, but these were considerably low in the intervention group. The frequency of zero adhesion (without adhesion band) occurred in five cases in the intervention group. In the control group, all cases had adhesion bands. Adhesion bands in the control group encroached into different parts of the abdomen (Fig. 1) while no encroachment was detected in intervention groups (Fig. 2). Intervention groups that received 1 per cent and 5 per cent extract presented 50 per cent (5 rats) and 40 per cent (4 rats) of adhesion type 1, but the control group showed 50 per cent adhesion type 2 (Table 3).

Fig. 1. Extensive adhesion bands encroached into different parts (control group).

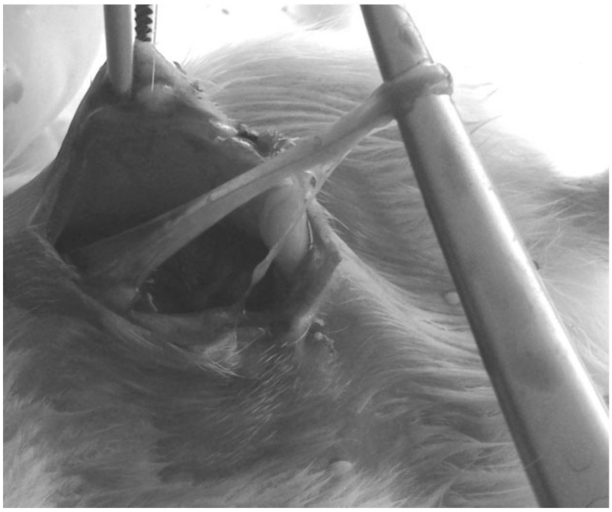

Fig. 2. Single adhesion bond on the both sides of the abdominal wall (intervention group).

According to the statistical analysis of data, macroscopic examination showed a significant difference between groups in terms of the adhesion formation ($P = 0.023$). According to the Mann–Whitney *U*-test for macroscopic data, adhesion formation in groups treated with 1 per cent and 5 per cent *S. marianum* extract was significantly lower than that of the control group ($P = 0.015$ and $P = 0.023$, respectively). There was no significant different between the extract-treated groups ($P > 0.05$). Histopathological examination showed significant differences between groups in the severity of fibrosis ($P = 0.028$) and inflammation ($P = 0.014$). For all groups, there was a significant difference between 1 per cent and 5 per cent between the extract-treated and control groups in term of fibrosis severity ($P = 0.003$ and $P = 0.002$, respectively) and inflammation ($P = 0.005$ and $P = 0.001$,

Table 3. Comparison of adhesion degree frequencies between the groups

Adhesion degree	1 per cent extract	5 per cent extract	Control
	n (frequency per cent)	*n* (frequency per cent)	*n* (frequency per cent)
0	3 (33 per cent)	3 (30 per cent)	0 (0 per cent)
1	5 (50 per cent)	4 (50 per cent)	2 (20 per cent)
2	1 (10 per cent)	2 (10 per cent)	5 (50 per cent)
3	0 (0 per cent)	1 (0 per cent)	2 (20 per cent)
4	1 (10 per cent)	0 (10 per cent)	1 (10 per cent)
Total	10 (100 per cent)	10 (100 per cent)	10 (100 per cent)

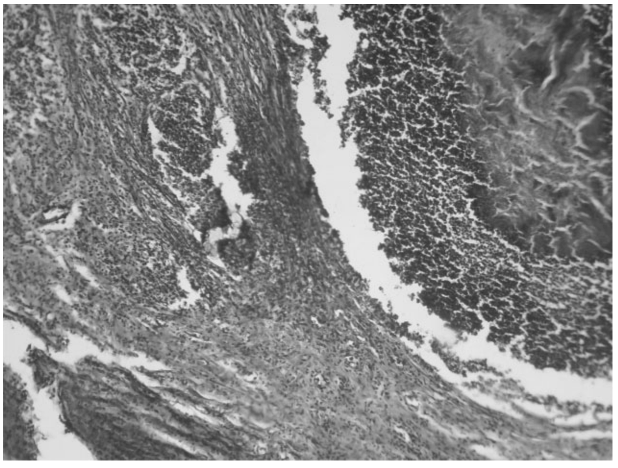

Fig. 3. Infiltration of inflammatory cells and areas of fibrosis during the histopathological examination of the control group (haematoxylin–eosin staining) (× 400).

respectively), but there was no significant difference between the *S. marianum* extract-treated group ($P > 0.05$). Figure 3 was taken from adhesion bands in the control group, which indicate fibrosis severity and inflammation.

Discussion

The aim of the present study was to investigate the preventive effect of *S. marianum* extract on post-operative adhesions. The results showed that extract was effective in the prevention of intraabdominal adhesions.

Intraabdominal adhesions are common following abdominal surgery, causing fatal complications, such as ileus, intestinal obstruction and infertility.[28] Adhesion following surgery is part of the natural healing process. Adhesion forms by any factor that leads to tissue hypoxia and ischaemia,[29] including oxygen-free radicals that are produced in the early phase of ischaemia and eliminate oxygen by reacting with it. The endogenous sources of superoxide and other free radicals include mitochondrial cytochrome oxidase and xanthine oxidase of endothelial cells.[30] Hydrogen peroxide and superoxide anion are toxic for some cells, such as endothelial cells, platelets and fibroblasts, because of extracellular cytolysis.[31] Cytolysis and peroxidation of membrane lipids might increase vascular permeability and exudate formation that initiate the adhesion process.[32] It seems that the prevention of free-radical formation in ischaemic tissue is by the antioxidant components of *S. marianum* extract.

Silymarin has no significant effect on unstimulated neutrophil mobility and phagocytic and chemotactic activity, but when neutrophils are stimulated, silymarin inhibits myeloperoxidase release. Silybin and neutrophil stimulation inhibit leukocyte mobility inhibitors.[33] A lower incidence of inflammation in the pathological samples of the intervention groups compared to the control group supports this. In a study antioxidant compounds have been shown to stimulate expression of connective tissue growth factor and inhibit collagen type I gene expression and their effects on regular grouping of collagen have been approved.[34]

Compounds with antioxidant capacity can be effective in wound-healing process. These compounds inhibit post-operative intra-abdominal adhesions by antiinflammatory activity, inhibition of complete fibrinolysis and collagen production and accumulation.[35] It seems that significant differences between groups in terms of fibrosis severity are because of this effect of *S. marianum*.

Although many studies have been performed on treatment of these complications, but few studies have been done on compounds with antioxidant activity. In a study by Klass *et al.*, the inhibitory effect of green tea extract on intraabdominal adhesion was examined in rats, and its inhibitory effect was attributed to the antioxidant properties.[16] In another study, lipid peroxidation inhibitors were shown to prevent peritoneal adhesions in rabbits.[6] Silymarin acts as an antioxidant and free-radical quencher, thus providing protection against chemical-induced peroxidation, which was supported by the findings in our study.[36]

In the present study, there was no significant difference between the 1 per cent and 5 per cent extract-treated groups. Further research on other doses of extract to determine the most effective dose are recommended. According to our results, *S. marianum* extract has antioxidant and antiinflammatory effects. It can be effective in healing abdominal cavity lesions and for the prevention of the adhesion process. Therefore, by conducting further clinical studies on drugs derived from *S. marianum* extract with the best dose, it could be used in the near future as a commercial drug in humans for the treatment of complications.

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Declaration of conflict of interest

All authors declare that they have no conflicts of interest.

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